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Monoclonal antibodies biosimilarity assessment using transient isotachophoresis capillary zone electrophoresis-tandem mass spectrometry

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Abbreviations: mAb, monoclonal antibody; CESI-MS, sheathless capillary electrophoresis-mass spectrometry; CZE, capillary zone electrophresis; AA, amino acid; t-ITP, transient isotachophoresis; HC, heavy chain; LC, light chain; CHO, Chinese hamster ovary; PTM, posttranslational modification

Out of all categories, monoclonal antibody (mAb) therapeutics attract the most interest due to their strong therapeutic potency and specificity. Six of the 10 top-selling drugs are antibody-based therapeutics that will lose patent protection soon. The European Medicines Agency has pioneered the regulatory framework for approval of biosimilar products and approved the first biosimilar antibodies by the end of 2013. As highly complex glycoproteins with a wide range of micro-variants, mAbs require extensive characterization through multiple analytical methods for structure assessment rendering manufacturing control and biosimilarity studies particularly product and time-consuming. Here, capillary zone electrophoresis coupled to mass spectrometry by a sheathless interface (CESI-MS) was used to characterize marketed reference mAbs and their respective biosimilar candidate simultaneously over different facets of their primary structure. CESI-MS/MS data were compared between approved mAbs and their biosimilar candidates to prove/disconfirm biosimilarity regarding recent regulation directives. Using only a single sample injection of 200 fmol, CESI-MS/MS data enabled 100% amino acids (AA) sequence characterization, which allows a difference of even one AA between 2 samples to be distinguished precisely. Simultaneously glycoforms were characterized regarding their structures and position through fragmentation spectra and glycoforms semiquantitative analysis was established, showing the capacity of the developed methodology to detect up to 16 different glycans. Other posttranslational modifications hotspots were characterized while their relative occurrence levels were estimated and compared to biosimilars. These results proved the value of using CESI-MS because the separation selectivity and ionization efficiency provided by the system allowed substantial improvement in the characterization workflow robustness and accuracy. Biosimilarity assessment could be performed routinely with a single injection of each candidate enabling improvements in the biosimilar development pipeline.

Introduction

Although monoclonal antibodies (mAbs) were introduced as treatments for disease in the late 1980s (muromonab-CD3 was approved in 1986), they currently represent the most rapidly growing category of therapeutic molecule.[1] Some of their properties naturally explain such success. For example, their therapeutic efficiency, reduction of side-effects, favorable pharmacokinetic (PK) and pharmacodynamics (PD) lead to an intensive appeal in research and development (R&D) activities regarding this type of protein for the last decade.[2] Currently more than 40 mAbs have been approved by regulation agencies, such as the US Food and Drug Administration (FDA) or the European Medicine Agency (EMA), and 30 additional candidates are currently in Phase 3 evaluation.[3,4] Their applications are mainly in the field of oncology, inflammation, immune mediated disorders and neurological diseases, including Alzheimer treatments.[5,6] Patents protecting the first generation blockbuster mAbs will expire in the next 5 y, giving the opportunity to many companies to produce "biogeneric versions." These copies are referred as biosimilars or in some cases follow-on biologics. A biosimilar is defined by the EMA as a biological medicinal product that contains a version of the active

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substance of an already authorized original biological medicinal product.[7] Recombinant mAbs are produced from proprietary cell lines; their structural complexity and important micro-variability induced by a given production process explain the greater difficulty that generic companies have in producing exactly reproduction of biologic molecules compared to small molecules. In the past few years, regulation agencies have worked toward establishment of guidelines that, while taking into account such limitations, determine critical quality attributes (CQAs) that must be in common between a reference mAb and its biosimilar suggesting that their immunogenicity, as well as their PK/PD, should not be significantly different. Those guidelines should help demonstrate similarity between a biosimilar candidate compared to the reference product. The work by EMA and FDA led to the publication of guidelines[7] and to the approval for marketing in the EU of a first biosimilar antibody (infliximab), thus paving the way for the whole product class.[8] Biosimilarity assessment includes extensive physicochemical characterization likewise PK and PD study, performed in a comprehensive manner. As different structural heterogeneities emerged from comparison of a biosimilar candidate with the reference molecule, more complementary studies should be performed in order to demonstrate the absence of toxicological and clinical effect.[9]

To perform a complete structural characterization in such a context, innovative analytical methodologies have a major role to play for the development and approbation of biosimilars, as well as for next generation mAbs. The possibility of obtaining a large variety of structural information over different aspects of the protein (e.g., AA sequence, posttranslational modifications, disulfide bonds, glycosylation), in an accurate and robust manner, is obviously necessary and could be an important asset for safety and product development. One illustration of that trend is the recent intensification of research work aiming to address new issues regarding mAbs characterization.[10-12] A crucial point that could significantly improve the biosimilar development pipeline is to obtain suitable structural information in order to quickly eliminate unsatisfying candidates. Mass spectrometry (MS) has progressively taken an important role in the characterization of therapeutic mAbs due to its outstanding selectivity, sensitivity and the possibility to obtain structural information. For example, characterization of therapeutic mAbs by MS has been applied to product and process development[13,14] and batch consistency assessment.[15] However, MS still needs to be used in concomitance with orthogonal analytical techniques to be able to focus on the different facets of the protein including mainly liquid chromatography (LC).[16-22] Recently, we described a method using capillary zone electrophoresis (CZE) coupled to tandem MS with a sheathless interface to perform the simultaneous characterization of several aspects of a protein in one injection, including AA sequence and posttranslational modifications (PTMs).[23]

The sheathless CE-ESI-MS interface, referred to as CESI-MS, has been developed based on an original design proposed by Moini *et al.*[24] Detailed description of this interface has been given by Haselberg *et al.*[25] The CESI system has been used to perform successful CE-ESI-MS experiments in proteomics,[26,27] metabolomics,[28] intact proteins,[25] therapeutic small molecules[29] and as an infusion platform,[30] and it demonstrated a drastically increased sensitivity compared to sheath-liquid CE-MS interfaces because of its low operating flow rates below 100 nL/min.

In the present work, we developed a transient isotachophoresis CE-ESI-MS/MS methodology, with the CESI-MS system, to first obtain the characterization of several mAbs. This extensive characterization over different levels of the protein, was performed in a single analysis of each sample. In a second time, each mAb was compared to a candidate biosimilar to establish if the methodology developed could be used to assess the biosimilarity between 2 samples. The comparison was made using the data from a single injection of each sample. Transient isotachophoresis (t-ITP) was introduced in this work: t-ITP is a preconcentration method widely used with CZE.[31] The use of t-ITP enables, in the case of bottom-up proteomics, use of sample concentrations similar to those required for nanoLC-MS/MS analysis.[26]

The mAbs included are trastuzumab, cetuximab and their respective candidate biosimilars. Trastuzumab is a humanized immunoglobulin gamma 1 (IgG-1) directed against the HER2/neu receptor overexpressed in around 20–30% of invasive breast cancer (HER2 positive) and more recently to treat HER2+ gastric cancer.[32,33] It was first approved as a therapeutic in the US and EU in 1998 and 2000, respectively. Trastuzumab is composed of 2 heavy chains (HC) and 2 light chains (LC) representing a total of 1328 AA. Trastuzumab HC bears an N-glycosylation consensus site on the asparagine (Asn) in position Asn-300[2] (**Fig. 1**). Cetuximab is human/murine chimeric IgG-1 directed against the epidermal growth factor receptor (EGFR) overexpressed in advanced-stage EGFR positive colorectal cancer. Cetuximab was approved in the US and EU in 2004 and 2005, respectively. It is composed of 2 HC and 2 LC as well for a total of 1326 AA and bears 2 N-glycosylation sites, both present on the HC. One is located on the Asn-299 and the other is present on the Asn-88[34] (**Fig. 1**). Note that mAbs' glycosylations are subject to extensive characterization and profiling because it significantly influences antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC).[35-37]

A tryptic digest of each sample was prepared using an adapted in-solution digestion protocol. The digests were then separated

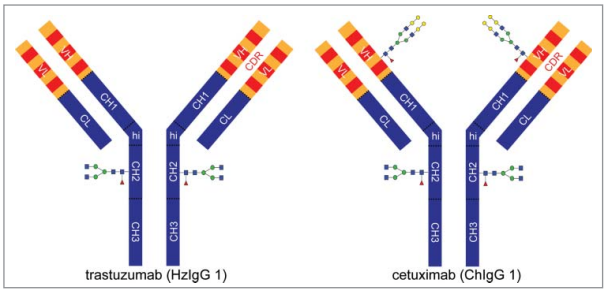


Figure 1. Schematic representation of trastuzumab and cetuximab. Blue parts of the protein represent the constant domain of the mAbs. Yellow parts of the scheme represent the variable domain of the mAbs and red parts represent the complementary determining region (CDR).

and characterized by t-ITP CESI-MS/MS. The peptide mapping was first performed using a peptide fragment fingerprinting (PFF) strategy similar to conventional bottom-up proteomics. Glycosylations were structurally characterized using MS/MS spectra and a semiquantitative profiling was established. Other posttranslational modifications (PTMs) of interest were investigated to complete the characterization of mAb samples. Finally trastuzumab and cetuximab CESI-MS/MS characterization data were compared to the data obtained for their respective biosimilar candidates in an equivalent perspective as required by regulation agencies to assess biosimilarity. This comparison was performed in order to determine if the developed t-ITP CESI-MS/MS methodology could be applied to demonstrate the biosimilarity between 2 mAbs in a robust and hastened manner, thereby improving biosimilars development pipeline.

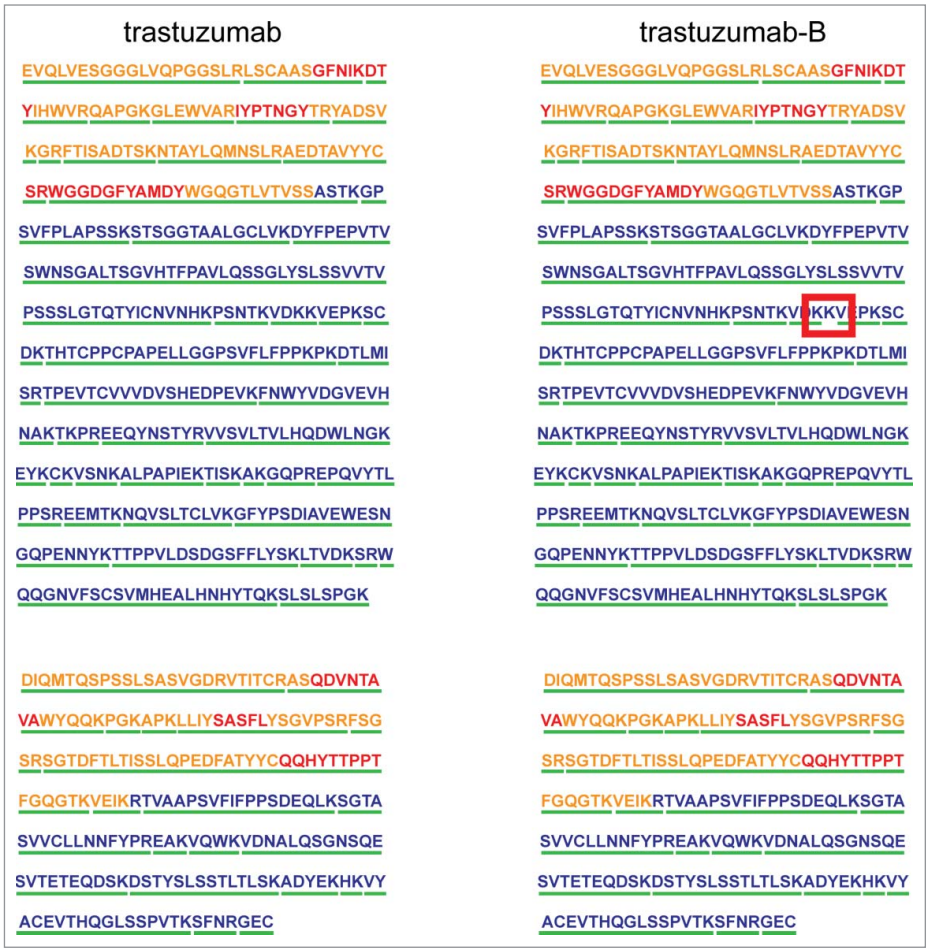


Figure 2. Sequence coverage obtained by CESI-MS/MS for trastuzumab (left-hand side) and trastuzumab-B (right hand side) showing only one amino acid (Lys-217) unidentified in the case of the candidate biosimilar. Experimental conditions: bare fused silica capillary (95 cm; 30 μm i.d.); sample mAbs tryptic digest 2.22 μM; injection volume 90 nL.

Results and Discussion

Amino acid sequences characterization

AA sequence is a key criteria for demonstrating the biosimilarity of a candidate. Indeed, along with other attributes, regulations require that the AA sequence of a candidate is proven to be the same as the reference product in order to be assimilated as biosimilar. Each mAb sample was digested by trypsin using an adapted digestion protocol in order to obtain a peptide mixture that could be characterized by CESI-MS/MS enabling demonstration/invalidation of the biosimilarity of 2 mAbs. Another aspect of the sample preparation is the source of the sample, reference mAbs (trastuzumab and cetuximab) in their commercial formulation were compared to 2 potential biosimilars (trastuzumab-B and cetuximab-B) in development that were sampled directly from the test bioreactor and purified, meaning that matrixes were significantly different. Sample preparation requires then cancelation of matrix effects, which then renders CESI-MS/MS data completely comparable.

For trastuzumab, the analysis of the tryptic digest using the CESI-MS/MS method allowed acquisition, in a single analysis, of 100% sequence coverage on both HC and LC while injecting only 32 ng of digested protein. In a previous work, we demonstrated the capacity to obtain the complete sequence coverage in a single analysis using CESI-MS/MS.[23] It is important to note, however, that significant improvement of the methodology enabled us to obtain the complete AA sequence characterization exclusively through the identification of peptides, without miscleavages nor posttranslational modifications, as emphasized in **Figure 2**. Trastuzumab-B was characterized using the same methodology. As shown in **Figure 2**, the AA sequence characterization of this biosimilar allowed demonstration of a complete AA sequence similarity to the reference protein except for HC lysine[217] (lys-217). Even by considering tryptic peptides with miscleavages, the AA lys-217 could not be confirmed, suggesting that this particular AA may be substituted in trastuzumab-B compared to the reference mAb. The interpretation of unassigned MS/MS spectra allowed us to demonstrate that the AA composing trastuzumab-B in position 217 was an arginine instead of a lysine, as illustrated by the spectra in **Figure 3**. Several ions pointed out this particular AA substitution reinforcing the confidence of this interpretation (**Fig. 3**). From a regulatory standpoint, the substitution of one AA does not fit with the highly similar paradigm, and thus trastuzumab-B would not be considered biosimilar by either the EMA or FDA.

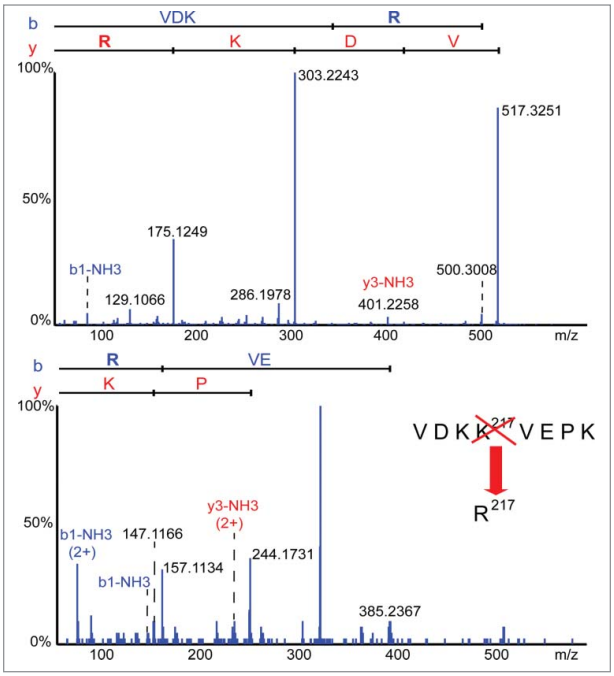


Figure 3. Raw MS/MS, extracted from trastuzumab-B CESI-MS/MS analysis, presenting fragmentation of ions m/z 517.3095 (1+) and m/z 314.6937 (2+) characterizing the amino acid substitution between trastuzumab and its candidate biosimilar. VDKK217VEPK for trastuzumab (G1m17 allotype) and VDKR217VEPK in the case of trastuzumab-B (G1m3 allotype).

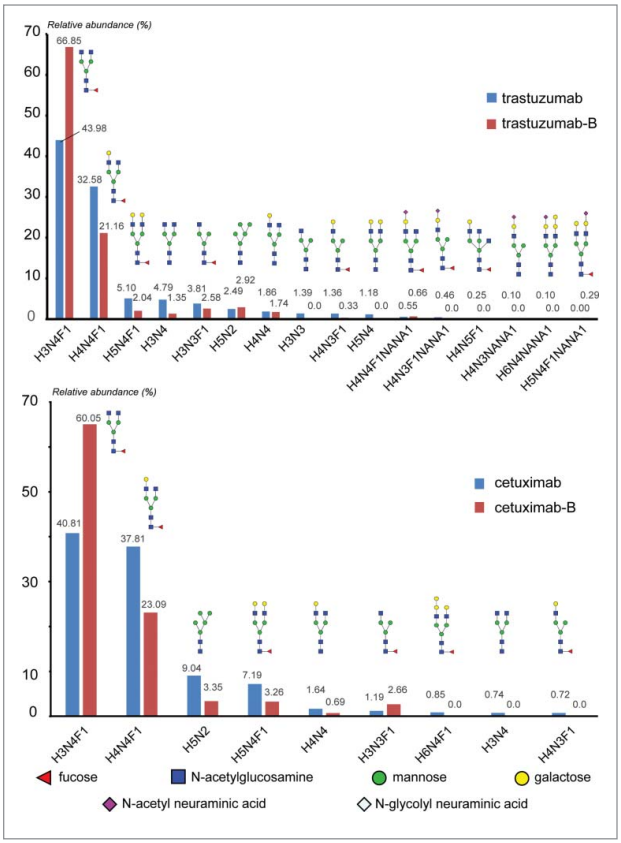


Figure 4. Glycoforms semiquantitative analysis results obtained through the CESI-MS/MS data for the Fc peptide of trastuzumab/trastuzumab-B and cetuximab/cetuximab-B. Glycoform profiling could be compared between original mAbs and their candidate biosimilar.

The same experiment was performed for cetuximab and cetuximab-B head-to-head. Once again, one single injection of cetuximab digest allowed the complete characterization of the AA sequence in this case as well through peptides without modifications nor miscleavages, demonstrating the robustness of the methodology. Cetuximab-B was produced in Chinese hamster ovary cells (CHO) in contrast to the reference product, which was produced in mouse myeloma cells (Sp2/0), but based on the same amino-acid sequence. The comparison of the CESI-MS/MS data, from unique injections of each sample, proved the complete equivalence of the AA sequence between cetuximab and its biosimilar candidate.

The experiment was repeated in 3 technical replicates and the same results were obtained in a single analysis of each mAb sample, demonstrating the reproducibility in term of characterization of the developed methodology. The CESI-MS/MS interface demonstrates its capacity to provide an impressive sensitivity, as it allows advanced characterization of the AA sequence using only 32 ng of digested mAbs. Even more, the MS/MS data exhibited the capacity to retrieve systematically more than 70% of the y/b fragment ions. In the case of trastuzumab, more than 90% of the y/b ions were obtained, which resulted into the possibility to unravel the totality of the AA organization over the variable domain. That trend in MS/MS spectra quality, even on a MS using a TOF analyzer enabling ions accumulation only on a reduced time, is explained by the excellent ionization efficiency when coupling CE to MS through sheathless interfacing. Indeed, the low flow rate induced by the electroosmotic flow (<40 nL/min) and the intrinsic characteristics of the CESI interface contribute to the formation of a nano electrospray (nanoESI). The nanoESI improves analyte ionization and reduces ion suppression effects, therefore impacting positively on MS/MS spectra quality.[30] The capacity to obtain the totality of the y/b ions, meaning by deduction the AA succession order, for the variable domain could be a huge asset in mAbs development because this part of the protein is directly responsible for antigen epitope recognition, and is therefore crucial for mAbs potency.

Glycosylation structural characterization and semiquantitative profiling

Glycosylation is a PTM that occurs naturally during excretion of antibodies from the expression system to the extracellular medium. It only represents ~3% of the total mass of the protein but is subject to extensive studies due to its significant influence on effector functions of mAbs, i.e., ADCC and CDC.

No glycan release was performed during the sample preparation and glycans could be characterized directly on the corresponding digested peptide. In the case of the trastuzumab/trastuzumab-B biosimilarity study, the injection of the reference mAb allowed identification of 16 different glycoforms borne by

trastuzumab. Alongside of glycans structural characterization, the semiquantitative glycoforms profiling was performed for trastuzumab and its biosimilar candidate as shown in **Figure 4**. To estimate glycoforms distribution, maximum intensities of each charge states of the glycopeptides were considered. The glycoprofiling established for the protein demonstrated the possibility to access lowly abundant glycans which is of interest in comparability and biosimilarity studies.

These results suggest the compatibility between the MS dynamic range and the CE loading capacity. Note also that the glycosylation profiling established is consistent to data available in the literature.[38] Even subtle glycoforms distribution differences between trastuzumab and the candidate biosimilar could be distinguished from the results. Trastuzumab-B was also shown to exhibit a rather different glycoforms distribution while carrying no glycans containing sialic acid (**Fig. 4**).

The cetuximab/cetuximab-B biosimilarity study exhibited different particularities regarding glycosylations. Cetuximab bears 2 N-glycosylation sites on each HC; the first one is located in the Fc/2 domain and common to all IgGs, while the second one in the Fd domain.[39] Focusing on the glycopeptide allowed us to distinguish glycans with regard to their glycosylation sites, which cannot be done when using methodologies involving glycans release. In this study as well, glycosylation profiles established demonstrated the identification of a significant number of glycosylation on each sites as shown in **Figure 4-B** and **Figure 5**. Significant differences in the glycosylation profile between cetuximab and its biosimilar candidate could be characterized, showing the applicability of the method to biosimilarity assessment (**Fig. 4-B**). Several cases of hypersensitivity to cetuximab were reported in the literature; the cause of that side-effect was related to the galactose-α-1,3-galactose present on the Fd glycosylation site of the protein.[40] For the marketed version of cetuximab produced in SP2/0 cells, 18 different glycoforms were identified, with 88% capped by at least one α-1,3-galactose residue, 23% capped by a N-glycolylneuraminic acid (NGNA) residue and traces of oligomannose. Development of a cetuximab biobetter should take into account that side-effect in order to prevent the occurrence of such glycosylation and provide an improved product, e.g., by production in CHO cells. Fd glycosylation profiles established from CESI-MS/MS data demonstrated that cetuximab-B would not be a suitable biosimilar candidate but a possible biobetter. Thus, the glycans identified on the Fd glycosylation site of cetuximab-B were significantly different from those present on the innovator mAb. The profile established for cetuximab-B therefore demonstrated that the abundance of glycoforms containing galactose-α-1,3-galactose could be significantly reduced (**Fig. 5**).

Highly sensitive glycosylation semiquantitation is often performed by removing the glycans using specific enzymes.[41] Results proved that the methodology used in this study additionally to reduce sample treatment, lowering the potentiality of artifacts, allows researchers to specifically locate the glycosylation sites and study them independently, with a sensitivity challenging already applied methods. The adopted strategy has allowed, concomitant with AA sequence characterization, to demonstrate/disconfirm the biosimilarity regarding the glycosylation profile between reference mAbs and their candidate biosimilar.

PTMs hot-spots comparison study

Other PTMs besides glycosylation must also be considered in biosimilarity assessment. PTMs referred as hot-spots are described in the literature as being induced by changes of the protein structure. Some of them classified as CQAs may influence the immunogenicity, the PK/PD of the protein which explains the analytical developments achieved to perform their characterization.[42,43] It is therefore necessary to prove that none of those PTMs are over represented in a biosimilar candidate, including N-terminal glutamine/glutamic acid cyclization leading to the formation of N-terminal pyroglutamic acid (pE), asparagine deamidation (deaN), methionine oxidation (oxiM) and aspartic acid isomerization (isoD) on various position depending on the considered mAb.[2] CESI-MS/MS data were also used to characterize the PTM hot-spots of the different mAbs samples prepared. PTMs occurrence levels were estimated from the data and finally compared between a biosimilar candidate and the corresponding originator antibody.

Data showed that every single PTM hot-spot monitored could be successfully characterized (**Table 1**). The use of electrophoretic separation is particularly pertinent to the characterization of PTMs. Indeed, PTMs induced significant change of electrophoretic mobilities between the modified digested peptides and its unmodified homologs. Therefore, those peptides could be separated by the electrokinetically driven separation of CE, from 0.5 min in the case of deaN to several minutes for pE. The use of CE as a separating technique is clearly of great interest. As emphasized in **Figure 6**, it was possible to separate the same peptide differing solely by the conformation of its aspartic acid. The separation in this particular modification allows additional information regarding the characterization to be obtained. Indeed, the isomerization of aspartic acid does not involve a mass change of the peptide and cannot be identified by MS using a time-of-flight (TOF) analyzer. Moreover, the ability to separate, upfront to MS

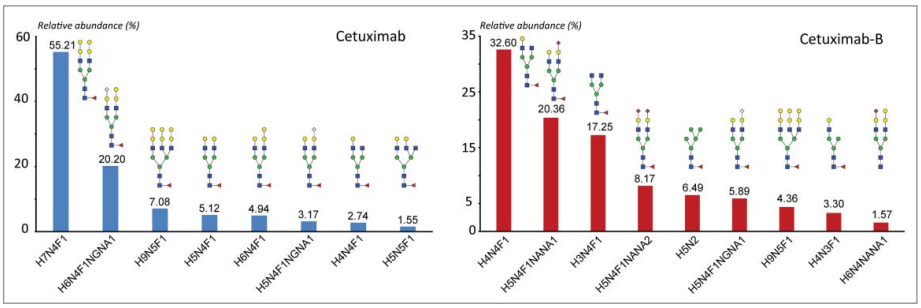


Figure 5. Glycoform semiquantitative analysis results regarding Fd domain glycosylation of cetuximab and cetuximab-B.

Table 1. Table summarizing the study regarding PTM hotspots occurrence levels for trastuzumab/trastuzumab-B obtained by CESI-MS/MS for each tryptic digest, modification levels (distribution% intact /% modification) were determined through signal intensity for each targeted PTM

			Trastuzumab distribution		Trastuzumab-B distribution	
position	**sequence**	**PTM**	**unmodif**	**modif**	**unmodif**	**modif**
1–19	**E**VQLVESGGGLVQPGGSLR	E^{1} / pE^{1}	98.2	1.8	97.3	2.7
51–59	IYPT**N**GYTR	N^{55} / $deaN^{55}$	89.4	10.6	92.3	7.7
99–124	WGG**D**GFYAMDYWGQGTLVTVSSASTK	D^{102} / $isoD^{102}$	84.9	15.1	86.0	14.0
252–258	DTL**M**ISR	M^{255} / $oxiM^{255}$	95.3	4.7	94.5	5.5
259–277	TPEVTCVVVDVSHE**D**PEVK	D^{272} / $isoD^{272}$	94.4	5.6	94.5	5.5
278–291	FNWYV**D**GVEVHNAK	D^{283} / $isoD^{283}$	92.0	8.0	96.5	3.5
374–395	GFYPSDIAVEWES**N**GQPENNYK	N^{387} / $deaN^{387}$	85.8	14.3	100.0	0.0
420–442	WQQGNVFSCSV**M**HEALHNHYTQK	M^{431} / $oxiM^{431}$	97.8	2.2	95.2	4.8
396–412	TTPPVLDS**D**GSFFLYSK	D^{404} / $isoD^{404}$	93.0	7.0	93.9	6.1
25–42	ASQDV**N**TAVAWYQQKPGK	N^{30} / $deaN^{30}$	96.1	3.9	96.0	4.0
150–169	VDNALQSGNSQESVTEQ**D**SK	D^{167} / $isoD^{167}$	64.0	36.0	91.6	8.4
170–183	**D**STYSLSSTLTLSK	D^{170} / $isoD^{170}$	86.6	13.4	92,9	7.1

analysis, a peptide that experienced chemical modifications from the same peptide without modification allows sensitivity to be maximized. In the case of co-migration, those peptides would compete against each other during the ionization process. Signal is improved by their separation and occurrence levels accuracy theoretically optimal. Specificity of CE separation enables the separation of digested peptides experiencing aspartic acid isomerization; that characteristic is particularly valuable as the characterization of such modification requires particular methods giving access solely to this information,[21] in contrast, here the study of aspartic acid isomerization could be incorporated within the framework of the overall primary structure characterization. The excellent MS/MS spectra quality obtained was particularly useful for PTM hot-spots characterization as it was possible to point out precisely the AA affected by the modification. PTM hot-spots semiquantitative measure was performed for each sample and occurrence levels were compared between the reference mAb and its respective candidate biosimilar as emphasized in **Table 1 and 2**. In this part, relative occurrence levels were estimated from the maximum intensities of the ions corresponding to the modified peptides compared to the abundance of the corresponding unmodified peptide.

In the context of the trastuzumab/trastuzumab-B biosimilarity study, results showed that endogenous deamidation due to proteolytic digestion conditions could be significantly controlled as several deamidation sites exhibited modifications levels below 7% and even 4% for $deaN^{55}$ (**Table 1**). Results demonstrated the capacity of this methodology to distinguish significant variation of modification levels. Therefore, results showed that isoD-170 (LC) abundance was superior to 13.4% in trastuzumab while its candidate biosimilar exhibited only 7.1% on this modification (**Table 1**). A similar trend could also be unraveled with the presented results for isoD-167 (LC). These high levels of modifications were correlated to the prolonged time the sample of reference trastuzumab had been stored. Regarding deaN, results of trastuzumab/trastuzumab-B showed dissimilarities for deaN-387 (HC) only, indeed trastuzumab-B exhibited less than 1% of this modification. OxiM results obtained from this study proved similar level of oxidation on the designated sites (**Table 1**).

Relative occurrence levels of PTM hot-spots were also studied and compared in the case of cetuximab/cetuximab-B.

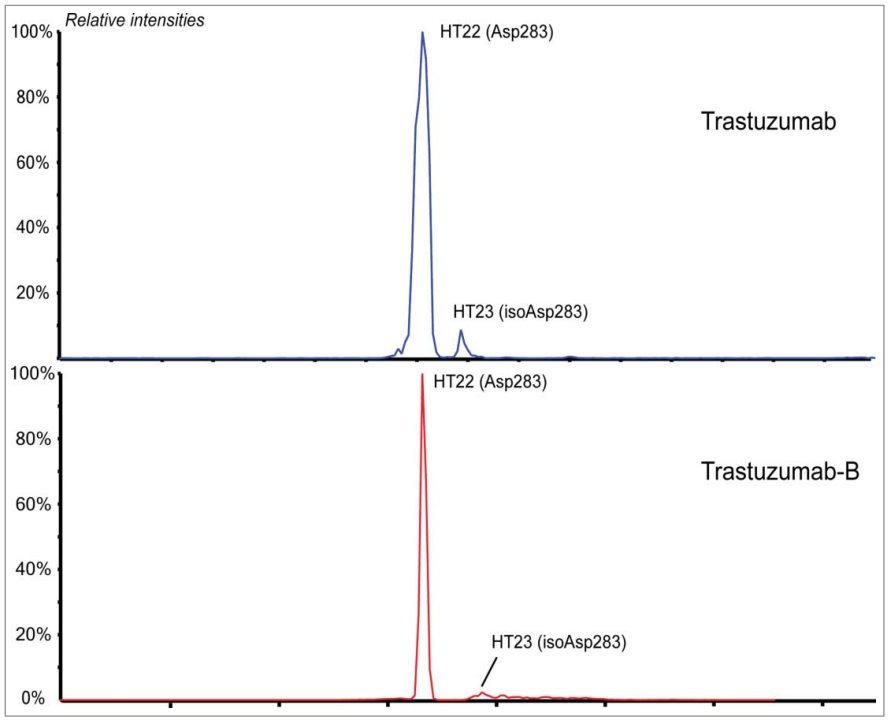


Figure 6. Extracted Ion Electropherogram (EIE) corresponding to m/z (839.408, 2+) for digested peptide HT22 (position 278–291) experiencing aspartic acid isomerization.

Table 2. Table summarizing the study regarding PTM hotspots occurrence levels for cetuximab/ cetuximab-B. Using the CESI-MS/MS data for each tryptic digest, modification levels (distribution% intact /% modification) were determined through signal intensity for each targeted PTM

			Cetuximab distribution		Cetuximab-B distribution	
position	sequence	PTM	unmodif	modif	unmodif	modif
1–5	**Q**VQLK	Q^{1} / pQ^{1}	100.0	0.0	100.0	0.0
150–212	DYFPEPVTVSW**N**SGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK	N^{161} / $deaN^{161}$	25.6	74.4	59.5	40.5
277–290	FNWYV**D**GVEVHNAK	D^{282} / $isoD^{282}$	95.2	4.8	96.3	3.7
373–394	GFYPSDIAVEWES**N**GQPENNYK	N^{386} / $deaN^{386}$	45.6	54.4	95.7	4.3
395–411	TTPPVLDS**D**GSFFLYSK	D^{403} / $isoD^{403}$	100.0	0.0	100.0	0.0
40–45	T**N**GSPR	N^{41} / $deaN^{41}$	87.8	12.2	94.7	5.3
150–169	VDNALQSG**N**SQESVTEQDSK	N^{158} / $deaN^{158}$	96.6	3.4	100.0	0.0

Results obtained were compiled in **Table 2**. As in the first study, results obtained allowed successful characterization of each of the PTMs hot-spots present on cetuximab. Results of the semiquantitative analysis regarding PTMs hot-spots occurrence were compared. Notably, both mAbs demonstrated specific characteristics supporting their similarity: N-terminal glutamine (Gln) cyclization was shown to be complete because the intact peptide could not be detected in samples of either mAbs. This trend could also be confirmed on the HC for $isoD^{403}$ and $deaN^{158}$ (**Table 2**); indeed those modifications exhibited absolutely no occurrence in cetuximab or its candidate biosimilar, illustrating the similarity of those proteins on such specific aspect. Cetuximab, as opposed to cetuximab-B, demonstrated slightly superior deamidation levels in the case of $deaN^{172}$ and $deaN^{386}$ for the HC and $deaN^{41}$ on the LC (**Table 2**), which were correlated with a longer conservation period for the reference mAb sample. However, these results illustrate the sensitivity of the method, which allows only minor variations in PTMs occurrence levels to be distinguished. Regarding the biosimilarity assessment of PTM hot-spots, results did not demonstrated overexpressed modifications for the biosimilar candidate compared to their respective innovator mAbs showing they could comply with the guidelines on this particular aspect.

The characterization strategy developed in this study involved the use of CESI-MS/MS analysis. Data obtained from only one injection of each sample tryptic digest allowed characterization of each mAbs simultaneously over several levels defining their primary structure: AA sequence, glycosylation structure/relative abundance and specific PTMs hot-spots. The purpose was to study the biosimilarity between 2 approved mAbs (trastuzumab and cetuximab) and 2 respective biosimilar candidates. Biosimilarity study results unraveled that in both cases, biosimilar candidates would hardly be considered as such according to current regulations. In the case of trastuzumab-B, one AA difference compared to the original mAb led to this conclusion, while in the case of cetuximab-B, major glyco-profile variations were highlighted. Among the biosimilarity assessments performed successfully for each mAbs, data collected during the CESI-MS/MS analysis pointed out specifically which attribute of the protein did not comply with the reference mAb giving precious information from an R&D perspective. For example, the difference of one AA for trastuzumab-B strongly supports the need of re-engineering of the vector to produce the right allotype.[44] However, in the case of cetuximab-B, each aspect of the primary structure of the mAb demonstrated the biosimilarity except for the glycosylation. Yet recent advances in mAbs glycoengineering permits optimization of the mAbs glycoform expression, thereby suppressing undesired glycosylations.[45,46]

In summary, the CESI-MS/MS strategy developed in the context of this study appeared to be fully compatible with use as an orthogonal technique concomitantly to the now commonly used analytical techniques for characterization of mAbs, biosimilar, biobetters and, more generally, biotherapeutic proteins.

Conclusions

We report here an innovative strategy involving CE coupled to high resolution tandem MS to characterize mAbs simultaneously over different angles of their primary structure, in order to perform biosimilarity assessments between approved mAbs (trastuzumab and cetuximab) and candidate biosimilars. Prior to analysis, samples were digested by trypsin using an adapted in-solution digestion protocol allowing improvement in digestion yield and a standardized final sample content compatible with CE separation. The analysis of each sample allowed establishment of the similarity/dissimilarities concerning AA sequence between several approved mAbs and their respective biosimilar candidates.

The sensitivity and MS/MS spectra quality obtained, mainly supported by the instrumental setting used in this work, enabled AA sequence characterization on an unprecedented level as 100% sequence coverage could be obtained in a single analysis through identification of only tryptic peptides without miscleavages or PTMs. Furthermore more than 70% of the y/b ions could be systematically retrieved, providing detailed structural data over this aspect of the protein. Along with this characterization, structural and semiquantitative analysis of the glycoforms could be performed, which demonstrated the capacity of the method to locate precisely different glycosylations sites. The glycoforms profiling obtained demonstrated the capacity of the established methodology to detect quite low abundance glycans, enabling the detection of potentially undesired glycoforms. Likewise the profiling accuracy was also highlighted as relative abundances obtained could be correlated to other methods. CESI-MS/MS data enabled subtle differences in glycoforms distribution between reference

mAbs and their candidate biosimilars to be distinguished, proving that the dynamic range achievable with CE-ESI-MS coupling is fully compatible with this type of analytical problem. Finally, specific PTMs hot-spots were characterized using the very same CESI-MS/MS data used to characterize mAbs samples previously; results demonstrated the capacity of the method to characterize precisely the targeted sites.

The sensitivity provided by the CESI-MS system allowed, in most cases, the precise identification of the AA affected by the PTMs even in the circumstances of weakly abundant modifications. CE separation specificity proved valuable because peptides with chemical modification, as weak as aspartic acid isomerization, could be separated, enabling their concomitant characterization. The results illustrated the capacity of the method to observe differences in modification levels between the reference mAb samples and their respective candidate biosimilars studied. Data obtained from CESI-MS/MS analysis established dissimilarities that existed among the candidate biosimilars compared to the approved mAbs, demonstrating the ability of the developed methodology to address such analytical issues in a structured and comprehensive way while easing sample treatment and reducing the number of experiment required. Furthermore, results permitted identification of precisely which aspects of the candidate antibody did not comply with their assignment as biosimilars providing important structural information that could be used to strategically plan subsequent R&D work. This study illustrates without ambiguity the possibility of using CE coupled to MS as an orthogonal technique, along with techniques routinely applied, to address complex analytical issues such as mAbs biosimilarity assessment, and points out more generally the capacity of this type of coupling to characterize biotherapeutic proteins.

Materials and Methods

Materials

Chemicals used were of analytical grade or high purity grade and purchased from Sigma-Aldrich (Saint Louis, MO, USA). Water used to prepare buffers and sample solutions was obtained using an ELGA purelab UHQ PS water purification system (Bucks, UK). Trastuzumab and cetuximab samples are the EMA approved products and formulation purchased from Roche (Penzberg, GE) and Merck KGaA (Darmstadt, GE) respectively. The biosimilar versions were produced at the Center d'Immunologie Pierre Fabre (Saint Julien en Genevois, FR) for analytical methods development. Marketed mAbs were stored at 4°C for one year before characterization while biosimilar versions was analyzed soon after their reception (approximately 2 months), otherwise all samples were stored at 4°C. RapiGest SF surfactant was purchased from Waters (Milford, MA, USA).

Sample preparation

A volume corresponding to 100 μg of protein were used. Samples were first diluted using milliQ water to a final concentration of 6.7 μg/μL. Samples were then diluted using 0.1% RapiGest surfactant to reach a final concentration of 3.35 μg/μL and heated to 40°C during 10 min. Dithiothreitol (DTT) was added to the sample to a final concentration of 25 mM. Samples were then heated for a 5 min incubation at 95°C. After being cooled to room temperature (RT), iodoacetamide (IDA) was added to the sample to a final concentration of 10 mM and samples were kept in the dark for 20 min to allow alkylation of cysteines (Cys). A volume of 1 μL of trypsin (0.5 μg/μL) was added to the sample which was left at room temperature for 3 h and another volume of 1 μL was added after this time. Digestion was performed overnight at 37°C. After digestion completion, 1% (v/v) formic acid (FA) was added to the samples, which were left at RT in order to cleave the surfactant. Samples were finally diluted to a final concentration of 2.2 μM of protein using ammonium acetate 50 mM (pH 4.0).

Capillary electrophoresis

The CE separations were performed with a PA 800 *plus* capillary electrophoresis system from Beckman Coulter equipped with a temperature controlled autosampler and a power supply able to deliver up to 30 kV. Hyphenation was carried out using a CESI prototype made available by Sciex separation (Brea, CA, USA). Prototype of bare fused-silica capillaries (total length 100 cm; 30 μm i.d.) with a characteristic porous tip on its final 3 cm supplied by Sciex separation, a second capillary (total length 80 cm; 50 μm i.d.) filled during experiments with BGE allows electric contact. New capillaries were flushed at 75 psi (5.17 bar) for 10 min with methanol, 10 min with 0.1 M sodium hydroxide, then 10 min with 0.1 M hydrochloric acid and water for 20 min. Finally, the capillary was flushed 10 min at 75 psi with 10% acetic acid, which is the BGE used for the separation. Hydrodynamic injection (410 mbar for 1 min) corresponding to a total volume of 90 nL of sample injected was used. Separations were performed using a voltage of +20 kV.

Mass spectrometry

For antibody characterization, the CESI system was hyphenized to a 5600 TripleTOF mass spectrometer (ABSciex, Darmstadt, Germany). The 5600 MS is equipped with a hybrid analyzer composed of quadrupoles followed by a time-of-flight (TOF) analyzer. ESI source parameters were set as follows: ESI voltage −1.75 kV, gas supplies (GS1 and GS2) were deactivated, source heating temperature 150°C and curtain gas value 5. Experiments were performed in Top15 information dependent acquisition (IDA), accumulation time was 250 msec for MS scans and 100 msec for MS/MS scans leading to a total duty cycle of 1.75 sec. Mass/charge (m/z) range was set to 100–2000 in MS and 50–2000 in MS/MS. Using those parameters, the mean resolution provided by the instrument is 40000 in MS (m/z 485.251) and 25000 in MS/MS (m/z 345.235).

MS/MS data analysis

Data obtained from the CESI-MS/MS experiments were analyzed using Peakview software (ABSciex, Darmstadt,

Germany). Tryptic peptides (without miscleavages or PTMs except cys carbamidomethylation) were determined theoretically from the mAbs' amino acid sequences available through literature.[23,47] Additional peptides were identified using Mascot search engine provided by Matrix science; tryptic cleavage rules were applied. Carbamidomethylation of cysteine (+57.02 Da), N-deamidation of aspartic/isoaspartic acid (+0.985 Da) or succinimide intermediate (−17.03 Da), methionine oxidation (+15.99 Da) and N-terminal glutamic acid cyclization (-17.02 Da) were selected as variable modifications. The mass tolerance for precursor ions was set to ± 5 ppm and ±0.05 Da for fragmentation ions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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